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TITLE: A NEW BIOMECHANICAL ROLE FOR INTERMEDIATE FILAMENTS IN THE CORTICAL CYTOSKELETON - Highlighting the role of intermediate filaments in supporting the plasma membrane and facilitating the mechanosensory function of the cytoskeletal network

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ABSTRACT:

In considering the role of intermediate filaments in cells and tissues, we have become used to the iconic images displaying (keratin) networks traversing the cytoplasmic space of the cell between the nucleus and plasma membrane. These (keratin) networks have prominent radial spokes and when disease-causing mutations collapsed these into aggregates then the catastrophic consequences for the biomechanical properties of the cell seemed conclusive. Here we propose that intermediate filaments apposed to the plasma membrane and linking desmosomes are a key structural element in the cytoplasmic intermediate filament network and its contribution to the biomechanical properties of the cell. This is because spokes without a network of circumferential struts collectively forming a belt apposed to and supporting the plasma membrane do not constitute a filament network with optimal mechanical properties. It would be like a bicycle wheel without a rim (Fig. 1). This rim comprises the actin-rich cortical network and a belt of force-resistant intermediate filaments running parallel to and linked to the plasma membrane. The missing component in our appreciation of keratin mutations linked to skin blistering disease is this belt of intermediate filaments that connects desmosomes beneath the plasma membrane apposed to the actin-rich cortex at the cell periphery. We discuss this as a universal principle applicable to all cytoplasmic intermediate filament networks in cells where their role(s) demands plasma membrane support.

INTRODUCTION TO THE HYPOTHESIS – THE WHEEL, THE SPOKES AND THE RIM:

The desmosome–intermediate filament network is essential to the integrity of tissues [1-3]. As a cell-cell junction, the desmosome provides mechanical strength, integrating each individual cell into its tissue environment [1, 4-6]. Intermediate filaments are essential to these biomechanical properties as revealed by the numerous diseases caused by components of the keratin-desmosome complex ([7-12]; <http://www.interfil.org>; see also www.arvcdatabase.info for desmoplakin mutations). The iconic view of the desmosome-intermediate filament complex is of filament bundles interacting perpendicular to individual desmosomes forming a series of radial spokes that link the plasma and nuclear membranes in epithelial cells [13-15]. Here we describe an additional element to the intermediate filament network that connects desmosomes laterally, providing sub-plasmalemmal connections between desmosomes (Fig. 2). The intracellular network of intermediate filaments therefore comprises two components: one forming the radial spokes physically connecting the desmosome to the nucleus, whilst the other provides a circumferential belt running parallel to and closely apposed to the plasma membrane interconnecting cell-cell attachment sites. The potential impact of these circumferential intermediate filaments is revealed through high-resolution light microscopy studies in combination with electron microscopy (Fig. 2) and lends support to a mechanosensory role for keratin filaments [16] given their links to both the plasma membrane and

nuclear membrane via nesprins. We propose that these circumferential intermediate filaments form struts between individual desmosomes collectively forming a belt that can both position and support desmosomes at the plasma membrane, as evidenced from studies on keratin-null embryos where desmosome positioning was lost [17]. We suggest their presence lends biomechanical support to the regions of plasma membrane between desmosomes, regions that are likely more sensitive to rupture in disease-based scenarios eg [8, 18]. This hypothesis is consistent with both the fragile [19, 20] and sparse network [21] hypotheses that explain the mechanistic basis to the skin blistering disease epidermolysis bullosa simplex.

EVIDENCE FOR THE SUBPLASMALEMMAL BELT OF IFS IN EPITHELIA

Desmosomes are cell adhesion structures [1] found in tissues and cells where significant mechanical forces need to be resisted, contributing to what has been termed “a mechanical syncytium” [22]. In epithelia, keratin intermediate filaments attach specifically to the desmosomal plaques and appear not to attach to other plasma membrane sites in the same focused way [14, 23-25]. They do so via a specific intermediate filament-binding domain in the C-terminal region of desmoplakin [26, 27]. In these tissues the radial organization of intermediate filaments emanating from the nucleus is prominent [14, 23-25]. In the suprabasal layers of the epidermis, the radial keratin bundles terminating at desmosomes are the most obvious feature, facilitated by the integration of new differentiation-dependent desmosomal proteins such as PKP1. These radial keratin bundles are not as prominent in the basal layer of the epidermis due to the lower desmosome density. It is here that the circumferential belt of keratin filaments will likely play a more significant biomechanical role [25, 28].

Observations of blastocysts derived from a knock-in mice producing YFP-labelled keratin 8 suggest a crucial role for interdesmosomal keratins in keratin network organization *in vivo*. The keratin network is the first cytoplasmic intermediate filament network to be formed during embryogenesis. Dotted keratin fluorescence first appeared at the plasma membrane of cell-cell borders. The dotted structures were positive for desmosomal markers and these were subsequently interconnected by keratin filaments running parallel to the plasma membrane ie a subplasmalemmal belt of keratin filaments (Fig. 2; [29]).

Studies on the *de novo* assembly [13] or reassembly of the keratin filament-desmosome networks in epithelial cells take advantage of the calcium dependency of desmosomal junctions (reviewed in [1]). Using rat carcinoma cells treated with calcium chelators, it was observed that keratin filament reassembly was initiated at the desmosomes [30]. Bundles of keratin filaments emanated from desmosomes, indicative of the spoke-like organization of the cytoplasmic filament network [31]. The formation of keratin filaments interconnecting desmosomal structures was a prominent feature of A431 cells transfected with a chimeric connexin-DSc1A construct [13] suggesting that this was sufficient both to nucleate desmosome assembly and to dock these intermediate filaments, their identity confirmed by immunoelectron microscopy.

A SUBPLASMALEMMAL BELT OF INTERMEDIATE FILAMENTS COMPROMISED IN SKIN BLISTERING PHENOTYPES

In a case of lethal acantholytic epidermolysis bullosa where intermediate filament connections to desmosomes have been prevented by compound heterozygote mutations in desmoplakin [32], then electron microscopy analyses confirmed that the plasma membrane “was stretched to its limits until intercellular cleavage occurred”, despite the fact that desmosomal connections remained. The same was reported for a recessive mutation in desmoplakin that removed the intermediate filament-binding domain [33]. These striking phenotypic details were also observed in the desmoplakin knockout mouse [4]. Basal cells of the epidermis have fewer desmosomes compared to the suprabasal layers [25, 28] with more exposed plasma membrane and relatively more prominent adherens junctions. Therefore the biomechanical contribution of the cortical belt of keratin filaments is more important to the basal cells. Interestingly, desmosomes were not split in half when cells were breaking apart, but ended up being partitioned to one of the two cells. Membrane blebs occurred in the adjacent cell

where desmosomes had been removed. In another reported case of a K14 knockout ([34]; see also [9]) cytolysis occurred in the subnuclear region of the basal cells in blister areas, hemidesmosomes were intact and retained cytoplasmic remnant, with intact nuclei floating amongst the debris from these lysed basal cells. Therefore for those tissues where desmosomes comprise 50% or more of the plasma membrane, such as the spinous layer keratinocytes [35], the preponderance of desmosomes compensate for the absence of a subplasmalemmal belt, but this is not the case in the basal cells.

Removal of desmoplakin prevents the attachment of the subplasmalemmal keratin filaments and the formation of the circumferential belt of keratin filaments in cultured keratinocytes ([4]. The biomechanical properties of the epidermis are compromised by the removal of desmoplakin evidencing the importance of the desmosome as an attachment site for these filaments [4]. These experiments were unable to determine the relative role played by the radial spoke versus the cortical belt of keratin filaments [4]. The spokes traverse the cytoplasmic space and correlate with localized nuclear deformation [36] supporting a role for keratin filaments in mechanotransduction via the nuclear intermediate filament cytoskeleton [37], links mediated by nesprins and the LINC complex [38]. Nevertheless, until we understand how to manipulate independently the spoke and rim components of the keratin network, their relative contribution to proposed mechanotransduction/mechanosensory functions will remain unclear [16, 36]. The belt of keratin filaments at the cell periphery is, however, formed first when desmosomes are present during development and before any spokes are apparent [29]. When we have sufficient experimental flexibility then we shall be able to explain basal cell cytolysis in the K14 knockout phenotype ([9, 34]) and test the hypothesis that the belt of intermediate filaments is an important biomechanical element.

EVIDENCE FROM NON-EPITHELIAL CELLS AND TISSUES FOR A SUBPLASMALEMMAL BELT OF IFs

Desmosomes are cell-cell junctions that are not restricted to epithelia [1]. They are also prominent features of the myocardium and Purkinje fibres [39] as well as meningeal cells [40]. In these examples, it is desmin and vimentin filaments respectively rather than keratins that attach to the desmosomes [39, 40]. The electron microscopy data obtained at the time demonstrated that these intermediate filaments were closely apposed and often parallel to the plasma membranes between intercalated disks (e.g., [41, 42]) and their identity as intermediate filaments was subsequently confirmed by immunoelectron microscopy. The EDTA-induced internalization of desmosomal structures, maintained the desmosomal-filament complex and in particular the filament interconnecting desmosomes (reviewed in [1]). Interestingly, the desmosomal structures are split in two when calcium is depleted as would be expected given the importance of calcium to the interdesmosomal adhesion (reviewed in [1]). These examples evidence that keratin can be exchanged for either desmin or vimentin, but the question is whether the desmosome is an absolute requirement for the cytoplasmic intermediate filaments to organize in a spoke and rim arrangement.

The term “subplasmalemmal” was used to describe filaments apposed to the lens fibre cell plasma membrane [43]. These comprise vimentin and the beaded filament proteins [44, 45] and the plasma membranes of lens fibre cells are enriched in plakoglobin, a key desmosomal component [46] as well as [47]. Lens fibre cells, however, have no cell-cell junctions with the morphological features of desmosomes (reviewed in [48]) and neither do they retain their nuclei, so for these specialized cells only the plasma membrane intermediate filament network persists. Compromising this intermediate filament network compromises the biomechanical properties of the lens itself [49]. We conclude therefore that there is considerable diversity in potential plasma membrane attachment sites for intermediate filaments. This needs to be seen in the context that there is also a blurring of the conventional categorization of cell-cell junctions on the basis of morphology, subcellular location and the tissues / cells in which they are expressed [50-52]. Therefore the plasma membrane attachment sites for cytoplasmic intermediate filaments is cell context specific, but the concept is generic.

The avian erythrocyte is just such an example and illustrates that even for a cell designed not to be incorporated into a tissue, the intermediate filament association with the plasma membrane is a prominent feature [53]. These cells remain nucleated unlike their mammalian counterparts and have an intermediate filament network comprising vimentin and synemin [54, 55]. Ankyrin has been implicated as a possible plasma membrane-located docking site [56, 57] and although the LINC complex was uncharacterized at that time, lamin B was identified as a potential nuclear envelope docking site for vimentin [58]. In arachnoidal tissues, vimentin connects to desmosomes [40], but this is not the full extent of potential plasma membrane docking sites, which also includes β 4-integrins [59]. Vimentin is therefore directly involved in both cell-cell and cell-extracellular matrix (ECM) interactions and their biomechanical functions in tissue architecture.

THE CONCEPT – PUTTING THE CIRCUMFERENTIAL BELT OF INTERMEDIATE FILAMENTS INTO THE NETWORK

Here we have advanced the concept that desmosomes are focus points for intermediate filaments by identifying the unappreciated element of the desmosome “wheel” – namely the circumferential belt of intermediate filaments. The spokes are prominent and the rim can be easily overlooked, but their intermediate filaments are resilient [60], bending or buckling in response to forces [61] in a phosphorylation dependent mechanism [62]. The rim also comprises intermediate filaments, running parallel to the plasma membrane and in the case of epithelia, for instance, interconnecting desmosomes. Whether these inter-desmosomal filaments interact directly or indirectly with the plasma membrane via linkers, such as plectin [63, 64], or other cytoskeletal elements, such as actin [20, 65], remains to be seen. The principle of an intermediate filament network associated with the plasma membrane is not epithelia specific, rather it appears to be common to all cells where membrane support is required, from avian erythrocytes, to cardiomyocytes and the lens fibre cells.

A fascinating consequence of the hypothesis proposed here is that the cortical interjunctional intermediate filament system integrates the filament-desmosome system into a single biomechanical unit - a tension-spoke network that links to the nucleus [36, 37]. With the belt of interjunctional filaments in place, the desmosome-filament network can not only help mitigate compressive and tensile forces experienced by the cell, but it can also act as a mechanosensor [16, 36], transducing these mechanical inputs from localized plasma membrane regions directly to the nucleus and the LINC complex [38, 66] via the keratin spokes. Just as the nuclear membrane and LINC complex provides the interface for cellular mechanosensory functions [67], so the plasma membrane and cortical cytoskeleton is the cell-cell/cell-ECM interface. Identifying the cortical subplasmalemmal belt of intermediate filaments therefore calls for a re-evaluation of intermediate filament function in normal and diseased tissues and their proposed mechanosensory role(s) given the discovery of this new element in the cytoplasmic intermediate filament network.

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FIGURES

Figure 1. The cartoon depicts how radial and cortical keratin filaments are distributed in a single plane of a basal keratinocyte at steady state (a), how these keratin filaments respond to mechanical pressure applied from the top (b) and how the absence of these filaments leads to cytolysis in between desmosomal adhesion sites upon mechanical stress (c).

The top panel shows a transverse section, the bottom panel a view of the horizontal sections demarcated in the panel above. (a) Presents the perinuclear keratin network that juts out into radial desmosome-anchored filaments ("spokes") in green. The subplasmalemmal interdesmosomal filaments are demarcated in blue ("struts"). (b) Mechanical pressure leads to cell flattening and expansion of the depicted section. This is coupled to tightening and extension of both radial and interdesmosomal filaments. (c) The presence of EBS mutant keratins results in abundant granule formation and is accompanied by loss of radial and interdesmosomal keratin while a thickened perinuclear network remains. The lack of the subplasmalemmal network results in rupture of the weakened cell cortex between desmosomes upon mechanical stress.

Figure 2. Subplasmalemmal keratins connect desmosomes in cultured cells (A-B'', E, F), developing embryos (C-D'') and epidermal keratinocytes (G) as part of an adaptable mechanosensitive membrane stabilizer.

(A) Survey fluorescence micrograph of live canine kidney MDCK-derived cell line MDc-2K18r producing YFP-labelled human desmosomal cadherin desmocollin 2 (depicted in red) and mRFP-labelled human keratin 18 (depicted in green). The image was recorded with an LSM710 confocal laser scanning microscope that is equipped with an Airy Scan unit. Note the keratin network surrounding the nucleus (N) that is connected through radial filament bundles to desmosomal cell junctions. The cell border at the lower left also depicts barely visible filaments that run in parallel to the plasma membrane connecting desmosomal adhesion sites. (B-B'') Superresolution structured illumination microscopy (OMX) reveals the interdesmosomal subplasmalemmal keratin system of two adjacent MDc-2K18r cells and its connectivity to radial filaments. (C-D'') Survey view (C) and higher magnification fluorescence images (D-D'') of a fixed murine blastocyst obtained from knock-in mice producing YFP-labelled keratins depicted in green (Schwarz et al., 2015) that were reacted with anti-desmoplakin antibodies (depicted in red). Images were recorded using an LSM710 confocal laser scanning microscope that is equipped with an Airy Scan unit. Note the accumulation of keratin 8 at desmoplakin-positive spots and the interconnecting keratin filaments that run in parallel to the adjacent plasma membrane. In addition, thin filaments extend toward the cell interior and the nucleus (N). (E, F) Electron microscopy of peripheral regions of adjacent MDCK cells. Note the two desmosome-associated filament systems, i.e. those that run in parallel to the plasma membrane and those that loop through the desmosomal plaque from the cell interior. (G) Electron micrograph of epidermal keratinocytes presenting dense interdesmosomal keratin bundles parallel to the plasma membrane. (H) Scheme depicting the arrangement of the two components in the keratin intermediate filament network. One connects the desmosomal cell-cell junctions via a subplasmalemmal belt and the other connects the desmosomes to the nucleus via radial spokes. Together they form an adaptable tension-spoke network that is important for maintaining mechanical equilibrium and sensing force changes.

Size bars, 10 μm in A and C; 1 μm in B-B'' and D-D''; 100 nm in E-G

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Figure 1

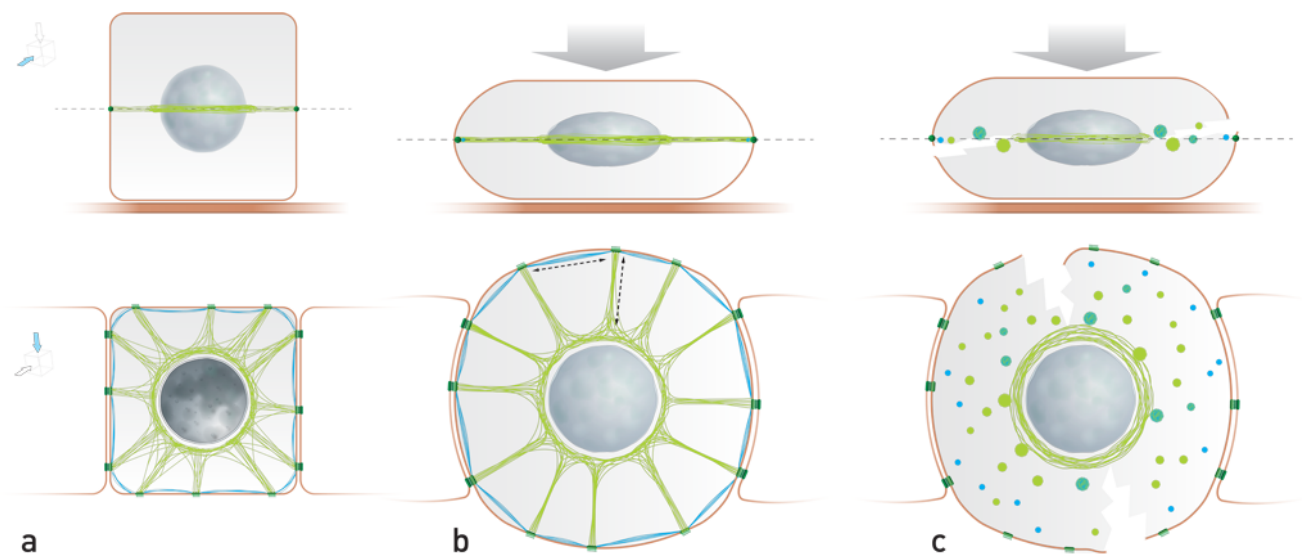


Figure 2

